

Interaction between Topically and Systemically Coadministered P-Glycoprotein Substrates/Inhibitors: Effect on Vitreal Kinetics

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ABSTRACT:

The objective of the present study was to investigate the effect of topically coadministered P-glycoprotein (P-gp) substrates/inhibitors on the vitreal kinetics of a systemically administered P-gp substrate. Anesthetized male rabbits were used in these studies. The concentration-time profile of quinidine in the vitreous humor, after intravenous administration, was determined alone and in the presence of topically coadministered verapamil, prednisolone sodium phosphate (PP), and erythromycin. The vitreal pharmacokinetic parameters of quinidine in the presence of verapamil [apparent elimination rate constant (λ_z), $0.0027 \pm 0.0002 \text{ min}^{-1}$; clearance (CL_F), $131 \pm 21 \text{ ml/min}$; area under the curve ($AUC_{0-\infty}$), $39 \pm 7.0 \mu\text{g} \cdot \text{min/ml}$; and mean residence time, $435 \pm 20 \text{ min}$] were significantly different from those of the control ($0.0058 \pm 0.0006 \text{ min}^{-1}$,

$296 \pm 46 \text{ ml/min}$, $17 \pm 3 \mu\text{g} \cdot \text{min/ml}$, and $232 \pm 20 \text{ min}$, respectively). A 1.7-fold decrease in the vitreal λ_z and a 1.5-fold increase in the vitreal AUC of quinidine were observed in the presence of topical PP. Statistically significant differences between the vitreal profiles of the control and erythromycin-treated group were also observed. Plasma concentration-time profiles of quinidine, alone or in the presence of the topically instilled compounds, remained unchanged, indicating uniform systemic quinidine exposure across groups. This study demonstrates an interaction between topically and systemically coadministered P-gp substrates, probably through the modulation of P-gp on the basolateral membrane of the retinal pigmented epithelium, leading to changes in the vitreal kinetics of the systemically administered agent.

Introduction

Drug delivery to the back of the eye through the systemic route is a challenging task (Hughes et al., 2005). The blood-retinal barrier (BRB), consisting of the inner and outer BRB, prevents access of xenobiotics from the systemic circulation into the back of the eye (Cunha-Vaz, 2004). The retinal pigmented epithelium (RPE), which constitutes the outer BRB, acts as a major barrier not only to systemically administered agents but also to compounds administered by the periorcular routes (Majumdar et al., 2009). The RPE is a single layer of hexagonal epithelial cells that forms the outermost layer of the retina and is separated from the choroid by Bruch's membrane (Cheruvu and Kompella, 2006). It carries out essential biochemical functions such as phagocytosis of photoreceptor outer segments, transport regulation between the photoreceptors and the choriocapillaries, and the uptake and conversion of the retinoids (Pitkänen et al., 2005). However, the RPE also presents a significant barrier to the

ocular penetration of many therapeutic agents (Majumdar et al., 2009).

After systemic administration, therapeutic moieties can easily diffuse out of the leaky choriocapillaries into the choroidal stroma (Dey et al., 2003; Duvvuri et al., 2003b; Cunha-Vaz, 2004). However, the tight junctions of the RPE regulate further diffusion of hydrophilic molecules and macromolecules from the choroidal stroma into the retina (Duvvuri et al., 2003b). Furthermore, P-glycoprotein (P-gp), a membrane-bound protein involved in the efflux of many hydrophobic molecules, expressed on the basolateral membrane of the RPE (henceforth referred to as RPE P-gp), plays a vital role in the RPE's barrier properties toward lipophilic compounds. The physiological role of this efflux protein is to protect the eye from harmful toxic substances. However, through this protective action, RPE P-gp severely limits ocular penetration of many systemically, as well as periorcularly, administered drugs (Majumdar et al., 2009). Thus, modulation of the activity of RPE P-gp could alter the penetration of substrates from the systemic circulation into the vitreous humor and subsequent elimination from the posterior chamber, which could significantly alter the ocular pharmacokinetic parameters of the systemically administered P-gp substrate.

The effect of a drug-drug interaction at the level of the RPE P-gp and its effect on ocular drug pharmacokinetics in vivo has attracted

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ABBREVIATIONS: BRB, blood-retinal barrier; RPE, retinal pigmented epithelium; P-gp, P-glycoprotein; PP, prednisolone sodium phosphate; HPLC, high-performance liquid chromatography; IPBS, isotonic phosphate buffered saline; IS, internal standard; AUC, area under the concentration-time curve; MRT, mean residence time; PHS, prednisolone hemisuccinate sodium; BAK, benzalkonium chloride.

attention only in recent years. There are literature reports of the effect of systemic/systemic, systemic/intravitreal, or intravitreal/intravitreal coadministration of inhibitors and model marker compounds on ocular pharmacokinetics (Duvvuri et al., 2003a; Senthikumari et al., 2008a,b). We recently demonstrated, for the first time, an interaction between topically and intravitreally coadministered P-gp substrates wherein the topically administered P-gp modulators altered the vitreal kinetics of an intravitreally administered P-gp substrate, quinidine (Majumdar et al., 2009).

The objective of the current research project was to determine the effect of topical P-gp inhibitors on the vitreal kinetics of a systemically coadministered P-gp substrate. For this purpose, two currently marketed ophthalmic formulations, erythromycin ophthalmic ointment (USP, 0.5%) and prednisolone sodium phosphate (PP) (USP, 1%), were administered topically. In addition, a 1% verapamil solution was also prepared for topical administration. Erythromycin and PP are routinely administered topically to control ocular infections and inflammation, respectively, and are well established P-gp inhibitors (Schwab et al., 2003; Majumdar et al., 2009). Verapamil is another well known P-gp substrate/inhibitor (Duvvuri et al., 2003a). Quinidine, which was used in our previous study as the marker compound (Majumdar et al., 2009), was used as a model P-gp substrate in this study also, and its vitreal and plasma pharmacokinetic parameters were determined.

Materials and Methods

Materials. Verapamil hydrochloride and quinidine hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO). PP ophthalmic solution (USP, 1%) and erythromycin ophthalmic ointment (USP, 0.5%), were procured from Butler Animal Health Supply (Dublin, OH). Ketamine hydrochloride and xylazine were purchased from Fort Dodge Animal Health (Fort Dodge, IA) and Lloyd Laboratories (Shenandoah, IA), respectively. Pentobarbital was obtained from Virbac AH, Inc. (Fort Worth, TX). HPLC-grade solvents and other chemicals (analytical grade) were obtained from Thermo Fisher Scientific (Waltham, MA). Microdialysis concentric probes (CMA/20; 20,000-Da cutoff, 0.5×10 mm polyarylethersulfone membrane and 14-mm shaft), used for sampling the vitreous chamber, were purchased from CMA Microdialysis, Inc. (North Chelmsford, MA).

Animals. New Zealand male albino rabbits were procured from Myrtle's Rabbitry (Thompson Station, TN). All of the animal experiments conformed to the tenets of the Association for Research in Vision and Ophthalmology statement on the Use of Animals in Ophthalmic and Vision Research and followed the University of Mississippi institutional animal care and use committee-approved protocols.

Microdialysis Probe Implantation and Recovery Studies. *In vivo probe implantation.* Rabbits (weighing 2–2.5 kg) were anesthetized using ketamine (35 mg/kg)-xylazine (3.5 mg/kg) administered intramuscularly and were maintained under anesthesia throughout the duration of the experiment (ketamine/xylazine administered intramuscularly every 40 min). Before probe implantation, the median ear artery of the rabbit was cannulated using a 24-gauge \times 3/4 in. SURFLO Teflon intravenous catheter with an injection plug (Terumo Medical Corp., Somerset, NJ) for collection of blood samples. After cannulation, microdialysis probe implantation was performed as described previously (Majumdar et al., 2009). In brief, eyes were proptosed, after the pupil was dilated with a 1% tropicamide solution, and a 22-gauge needle was carefully inserted into the vitreous chamber. The point of insertion was approximately 3 mm below the corneal-scleral limbus. The needle was retracted, and the vitreal probe was implanted immediately. The position of the probe was adjusted so that the semipermeable membrane was in the mid-vitreous section. The probes were continuously perfused with sterile isotonic phosphate-buffered saline (IPBS) (pH 7.4) at a flow rate of 2 μ l/min using microdialysis pump control module and syringe drives (Bioanalytical Systems, Inc. Mount Vernon, IN). After probe implantation, animals were allowed to stabilize for a period of 2 h before drug administration. Vitreal samples were collected every 20 min for a period of 9 h. Samples were collected in microcentrifuge tubes and stored at

–20°C until further analysis. At the end of the study, animals were euthanized with an overdose of sodium pentobarbital administered through the marginal ear vein. A limitation of the anesthetized rabbit model, accompanied with microdialysis, is that only a limited number of terminal elimination half-lives may be reached, particularly for compounds with long half-lives, which could affect half-life calculations. However, considering the goals of this research project, which was to investigate whether the vitreal elimination kinetics are altered, this model and experimental protocol serve the purpose.

In vitro probe recovery. Probe recovery values were determined by placing the probe in an IPBS solution, pH 7.4, at 37°C, containing a known concentration of quinidine. The probe was continuously perfused with sterile IPBS at a flow rate of 2 μ l/min, and the dialysate samples were collected every 20 min. Recovery of quinidine was calculated by using eq. 1:

$$\text{Recovery}_{\text{in vitro}} = C_d/C_s \quad (1)$$

where C_d is the quinidine concentration in the dialysate and C_s is the quinidine concentration in IPBS. The actual concentrations of quinidine in the vitreous humor during the *in vivo* pharmacokinetic study were calculated using eq. 2:

$$\text{Quinidine concentration in the vitreous humor} = C_q/\text{Recovery}_{\text{in vitro}} \quad (2)$$

where C_q is concentration of quinidine in the dialysate. In all of the studies, $\text{Recovery}_{\text{in vitro}}$ was determined before and at the end of *in vivo* experiment and average $\text{Recovery}_{\text{in vitro}}$ values were used to determine the actual concentration of quinidine in the vitreous humor.

Drug Administration. After the probe stabilization period, quinidine hydrochloride, formulated in propylene glycol-IPBS (40:60, pH 7.4), was administered intravenously through the marginal ear vein (5 mg/kg b.wt.). Vitreal kinetics of quinidine after intravenous administration was studied alone (control) or in the presence of the topically applied substrates/inhibitors; verapamil (1%, pH 6.0), erythromycin 0.5% ophthalmic ointment, and PP (1%). Four animals were studied on each treatment day, and the animals were dosed sequentially by groups (control followed by inhibitor groups). Verapamil and PP (100 μ l) were instilled in the conjunctival sac 2, 4, and 6 h after intravenous quinidine administration. In the case of the erythromycin study, a single dose of 0.5 mg of erythromycin (100 mg of ointment) was applied in the conjunctival sac 2 h after the intravenous quinidine injection. Verapamil solution was prepared by dissolving verapamil hydrochloride in sterile IPBS (pH 6.0) to yield a 1% solution; PP and erythromycin ophthalmic formulations were used as such. Verapamil and PP were dosed three times on the basis of data from and as a continuation of our previous work (Majumdar et al., 2009), wherein we demonstrated the effect of topical inhibitor on the vitreal kinetics of an intravitreally administered substrate. Erythromycin was dosed only once because an ointment formulation was used.

In our previous publication (Majumdar et al., 2009), to delineate the effect of the vehicle on the ocular tissue barrier properties we had studied the vitreal kinetics of intravitreally administered fluorescein (marker compound to monitor the integrity and tightness of blood-retinal barrier), alone or in the presence of topically coadministered verapamil (1% w/v, vehicle-IPBS, pH 6.0). The vitreal pharmacokinetic parameters of fluorescein remained unchanged in the presence of topically coadministered verapamil (Majumdar et al., 2009), indicating that the tightness and integrity of the blood-retinal barrier were not altered. None of the other components of the vehicle would interact with P-gp. Because the vehicle used in the current study is the same, a separate vehicle control was thus not included.

Vitreal microdialysis samples were collected every 20 min for a period of 9 h. Blood samples (0.5 ml) were also collected concurrently, in both control and inhibition study groups, from the catheter implanted in the central ear artery of each rabbit. Blood samples were drawn and then collected in heparinized vials at approximately 10, 15, 30, 60, 90, 120, 240, 360, and 480 min after intravenous quinidine administration. The exact time of blood collection was noted, and a plasma concentration-time profile was constructed accordingly. Plasma was separated from the whole blood by centrifugation at 13,000 rpm for 10 min at 4°C (accuSpin Micro 17R; Thermo Fisher Scientific) and was stored at –20°C until further analysis.

Distribution of Prednisolone/PP and Erythromycin in the Ocular Tissues after Topical Administration. In a separate set of studies, after the probe stabilization period, PP and erythromycin ophthalmic ointment were applied topically in the conjunctival sac of the rabbit eye at 2, 4, and 6 h and at 2 h, respectively. At the end of 9 h after quinidine administration, animals were euthanized, eyes were enucleated, and ocular tissues were collected and analyzed for drug content. No further attempts were made to understand the time course of levels of inhibitors in plasma, vitreous, and other ocular tissues after topical application.

Enzymatic Conversion of PP to Prednisolone. Prednisolone rather than PP, a phosphate ester of prednisolone, is known to interact with P-gp. Therefore, bioreversion rates of PP to prednisolone were determined in the aqueous humor, vitreous humor, lens, RPE/choroid, and iris-ciliary body following previously described procedures (Majumdar et al., 2009; Majumdar and Srirangam, 2009). Studies were performed in triplicate at 37°C in a shaking water bath (75 reciprocations/min). PP stock solution (100 μ l) was added to the required volume of the tissue homogenates and aqueous and vitreous humor to obtain a final PP concentration of 5 μ g/ml. The protein content in the tissue homogenates was determined using the Bradford method (Bradford, 1976). At predetermined time points, 100- μ l aliquots were withdrawn, and an equal volume of ice-cold methanol was immediately added to the sample to arrest the enzymatic degradation process. Stability of PP (5 μ g/ml) in IPBS was also studied as a control, and the values were subtracted from the overall reaction rates to estimate the enzyme-mediated degradation rates. The bioreversion rates were normalized to the protein content in the tissue homogenates.

Analytical Procedures. Ocular tissue collection and sample preparation. With respect to studies involving ocular distribution of PP and erythromycin after topical application, vitreous and aqueous humor were collected as described previously (Majumdar et al., 2009). Ocular tissues, such as iris-ciliary bodies, lens, and retina/choroid, were weighed and homogenized in ice-cold IPBS or methanol using a Tissuemiser (Thermo Fisher Scientific). Homogenates were further diluted with an ice-cold acetonitrile-methanol (50:50) mixture, centrifuged, and analyzed for drug content. Centrifugation was performed at 13,000 rpm for 20 min at 4°C. Aqueous and vitreous humor samples were used as such or diluted with methanol and taken for analysis. The extraction efficiency of PP and erythromycin from ocular tissues was >95%.

HPLC analysis. Microdialysis and plasma samples were analyzed for quinidine content using a reverse-phase HPLC procedure as described previously (Duvvuri et al., 2003a). The HPLC system comprised a Waters 717 Plus autosampler, a Waters 2475 multi λ fluorescence detector, a Waters 600 controller pump (Waters Corporation, Milford, MA), and an Agilent 3395 integrator (Agilent Technologies, Santa Clara, CA). A Symmetry C18 4.6 \times 250-mm column was used, and the mobile phase consisted of 20 mM phosphate buffer (pH 2.5) with 12% acetonitrile at a flow rate of 1 ml/min. Excitation and emission wavelengths were set at 250 and 440 nm, respectively. Microdialysis samples were directly injected into the high-performance liquid chromatograph. Plasma samples were analyzed after dilution with an ice-cold acetonitrile-methanol (50:50) mixture and centrifugation. For quantification of quinidine in microdialysis samples, calibration standards (2–200 ng/ml) were prepared in IPBS. For analysis of quinidine in plasma samples, a calibration curve (15–3000 ng/ml) was prepared by spiking the blank rabbit plasma with known concentrations of quinidine in IPBS, followed by protein precipitation with an acetonitrile-methanol (50:50) mixture. The standard curves generated coefficient of determination (r^2) values greater than 0.9999. The limits of quantification of quinidine in the microdialysis and plasma samples were 2 and 10 ng/ml, respectively. The percent relative S.D. (intraday and interday) for quinidine quality control samples in IPBS and rabbit plasma were less than 2 and 5.8%, respectively. Quinidine samples were analyzed in a single run or in groups. Quality control samples in IPBS (2, 5, 15, 100, and 160 ng/ml) and plasma (spiked concentration, 25, 75, 1000, and 2000 ng/ml) were always included in the runs after every 25 to 30 samples. The accuracy (intraday and interday) at different concentrations were within a range of 99.9 to 101.4% and 98.0 to 105.0% for quality control samples in IPBS and rabbit plasma, respectively. Prednisolone and PP contents in the bioreversion and ocular disposition studies were determined using a reverse-phase HPLC procedure as described previously with minor modification (Musson et al., 1991). Analysis was performed using a Waters 2487 Dual λ Absorbance Detector. The mobile phase consisted of isopropanol and 0.2% v/v *o*-phosphoric acid in deionized

water (25:75, pH 3.0). A YMC-Pack J'sphere ODS-M80 (4.6 \times 250 mm) column was used for separation, and the flow rate and wavelength (λ) were set at 0.8 ml/min and 245 nm, respectively.

Liquid chromatography/mass spectrometry analysis. Erythromycin contents in the ocular tissues, including vitreous and aqueous humor, were determined using liquid chromatography/mass spectrometry (liquid chromatography-electrospray ionization-time of flight). Primary stock solutions of erythromycin and the internal standard (IS), roxithromycin, were prepared in 100% methanol and secondary stock solutions were prepared in IPBS. A calibration curve was prepared by spiking known concentrations of erythromycin in blank ocular tissues, vitreous humor, and aqueous humor. The samples were allowed to stand for 30 min and then were homogenized in methanol using a Tissuemiser to produce calibration standards (10–500 ng/ml) of erythromycin.

The liquid chromatograph used was an Agilent series 1100 (Agilent Technologies) comprising the following modular components: quaternary pump, a vacuum solvent microdegasser, and an autosampler with 100-well tray. The mass spectrometric analysis was performed by using a liquid chromatography time-of-flight mass spectrometer (model G1969A; Agilent Technologies) equipped with an electrospray ionization source. All acquisitions were performed under positive ionization mode with a capillary voltage of 3500 V. Nitrogen was used as the nebulizer gas (30 psig) as well as the drying gas at 11 l/min at a temperature of 350°C. The voltages of the photomultiplier tube, fragmentor, and skimmer were set at 850, 100, and 60 V, respectively. Full-scan mass spectra were acquired from m/z 200 to 1000. Data acquisition and processing was done using Analyst QS software (Agilent Technologies).

Separation was achieved on a Synergi Hydro-RP column (100 \times 2.0-mm i.d., 4 μ m particle size; Phenomenex, Torrance, CA). The column was equipped with a guard column (Supelco, Bellefonte, PA). The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) at a flow rate of 0.3 ml/min, with the following gradient elution: 0 min, 75% A/25% B to 20% A/80% B over 10 min. Each run was followed by a 5-min wash with 100% B and an equilibration period of 11 min with 75% A/25% B. The total run time for analysis was 10 min. Ten microliters of the sample was injected, and peaks were assigned with respect to the mass of the compounds and comparison of the retention times. The limit of detection of erythromycin was 0.05 ng/ml, and each sample contained 100 ng/ml IS. This method involved the use of the $[M + H]^+$ ions in the positive ion mode with extractive ion monitoring. In the positive ion mode, the protonated species $[M + H]^+$ at m/z 734.4680 for erythromycin and 837.5324 for IS were observed.

Data analysis. Noncompartmental pharmacokinetic analysis was performed for the quinidine concentration-time profile in the vitreous humor using Win-Nonlin (version 5.2; Pharsight, Mountain View, CA). The pharmacokinetic parameters were calculated using the formulae described earlier (Majumdar et al., 2009). In brief, terminal slopes of the vitreous concentration-time profile were estimated by log-linear regression, and the apparent elimination rate constant (λ_z) was then derived from the slope. Elimination half-life ($t_{1/2}$) was calculated from the equation: $t_{1/2} = 0.693/\lambda_z$. The area under the vitreal concentration-time curve ($AUC_{0-\infty}$) and the area under the moments curve ($AUMC_{0-\infty}$) were estimated by the trapezoidal rule. The mean residence time (MRT) was calculated using the equation $MRT = AUMC_{0-\infty}/AUC_{0-\infty}$. The total clearance was calculated as $CL = Dose/AUC_{0-\infty}$. The peak vitreal concentration (C_{max}) and the time to reach the peak concentration (t_{max}) were determined from the observed values.

The time course of plasma quinidine concentration, after a single systemic dose was best described by a two-compartment open model as expressed in eq. 3:

$$C = Ae^{-\alpha t} + Be^{-\beta t} \quad (3)$$

where A and B are zero-time concentration coefficients, t is the time (minutes), and α and β are the disposition rate constants of the initial and terminal phases, respectively. The rate constant of drug transfer from the apparent peripheral to the apparent central compartment (k_{21}), elimination rate constant from the apparent central compartment (k_{10}), and rate constant of quinidine transfer from the apparent central compartment to the apparent peripheral compartment (k_{12}) were calculated as described previously (Majumdar et al., 2009). Data obtained were analyzed statistically using one-way analysis of variance followed by a post hoc Dunnett's test for multiple comparison (JMP software;

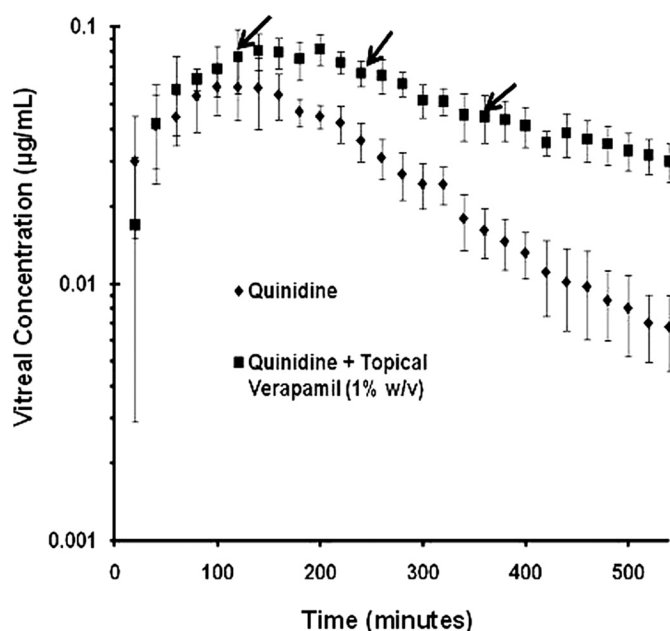


FIG. 1. Vitreal concentration-time profile of quinidine (5 mg/kg, systemic administration) alone (control) or in the presence of topically coadministered verapamil (1% w/v; 100 µl administered at 2, 4, and 6 h after quinidine administration). Arrows indicate time of inhibitor administration. Data points represent mean \pm S.D. of four determinations.

SAS Institute, Cary, NC). Homogeneity of variances between the groups was checked using Bartlett's test before analysis of variance was performed. Results were considered statistically significant if $p \leq 0.05$.

Results

Effect of Topically Applied Verapamil on the Vitreal Kinetics of Systemically Administered Quinidine. The vitreal concentration-time profile of quinidine after systemic administration (5 mg/kg) alone (control) or in the presence of topically administered verapamil is illustrated in Fig. 1, and the pharmacokinetic parameters are provided in Table 1. Verapamil HCl displayed low solubility at pH 7.4 (1 mg/ml), and, therefore, solutions were prepared in sterile IPBS at pH 6.0. With respect to the vitreal pharmacokinetic parameters of intravenously administered quinidine, topically instilled verapamil (100 µl of 1%, w/v), administered at 2, 4, and 6 h after systemic quinidine administration, produced a 1.9-fold increase in the MRT (from $232 \pm$

20 to 435 ± 20 min), a 2.2-fold decrease in the λ_z (from 0.0058 ± 0.0006 to 0.0027 ± 0.0002 min $^{-1}$) and a 2.3-fold increase in the $AUC_{0-\infty}$ of quinidine in the vitreous humor.

Effect of Topically Applied PP on Vitreal Kinetics of Systemically Administered Quinidine. PP is a phosphate ester of prednisolone and is currently marketed in the United States as an ophthalmic anti-inflammatory formulation. Therefore, the effect of topically administered PP on the vitreal kinetics of systemically administered quinidine (5 mg/kg) was investigated (Fig. 2). One hundred microliters of a 1% w/v PP solution was instilled at 2, 4, and 6 h after quinidine administration. Table 1 presents the vitreal pharmacokinetic parameters of quinidine. Topical PP coadministration produced a 1.7-fold decrease in λ_z (from 0.0058 ± 0.0006 to 0.0035 ± 0.0001 min $^{-1}$), a 1.7-fold increase in $t_{1/2}$, and a 1.3-fold increase in the MRT. Moreover, a 1.5-fold increase in the vitreal $AUC_{0-\infty}$ of quinidine, compared with that of the control, was observed when PP was coadministered.

Effect of Topical Erythromycin on Vitreal Kinetics of Systemically Administered Quinidine. Vitreal kinetics of intravenous quinidine (5 mg/kg) was also studied either alone or in the presence of topically administered erythromycin in the form of the marketed erythromycin ophthalmic ointment. In this set of studies, 100 mg of the ointment (0.5% w/w) was applied topically 2 h after systemic quinidine administration. Figure 3 depicts the vitreous concentration-time profile of quinidine alone or in the presence of topically administered erythromycin. The vitreal pharmacokinetic parameters are presented in Table 1. In the control group, the λ_z for quinidine was found to be 0.0058 ± 0.0006 min $^{-1}$ whereas in the presence of topical erythromycin, it was found to be 0.0040 ± 0.0002 min $^{-1}$. A corresponding 1.5-fold increase in the vitreal elimination half-life ($t_{1/2}$) was also observed. In addition, a significant increase in the $AUC_{0-\infty}$ and MRT of quinidine in the vitreous humor was evident.

Plasma Concentration-Time Profile of Quinidine in the Presence and Absence of Topically Applied Inhibitors. Plasma kinetics of systemically administered quinidine (5 mg/kg), alone or in the presence of topically coadministered verapamil, PP, and erythromycin was determined and compared with evaluate the effect of topical inhibitors on plasma kinetics of quinidine and to ensure uniform systemic exposure in all study groups. Figure 4 presents the plasma concentration-time profiles of quinidine alone or in the presence of verapamil, PP, and erythromycin, respectively. Plasma concentration-time data for quinidine, alone or in the presence of the inhibitors, were best explained by a two-compartment open model based on Akaike

TABLE 1

Vitreal pharmacokinetic parameters of systemically administered quinidine (5 mg/kg) alone or in the presence of topically coadministered verapamil, prednisolone sodium phosphate ophthalmic solution (USP, 1% w/v), and erythromycin ointment

Verapamil and prednisolone sodium phosphate were administered at 2, 4, and 6 h after systemic quinidine administration. A single dose of erythromycin ophthalmic ointment (100 mg) was applied at 2 h after quinidine administration. A single control group (quinidine) was compared with each treatment group. Values represent means \pm S.D. ($n = 4$).

Kinetic Parameters	Quinidine	Quinidine + Verapamil (1% w/v)	Quinidine + Prednisolone (1% w/v)	Quinidine + Erythromycin (0.5% w/w)
λ_z (min $^{-1}$)	0.0058 ± 0.0006	$0.0027 \pm 0.0002^{***}$	$0.0035 \pm 0.0001^{**}$	$0.0040 \pm 0.0002^{**}$
$t_{1/2}$ (min)	120 ± 14	$256 \pm 24^{***}$	$198 \pm 21^*$	$173 \pm 11^*$
t_{max} (min)	115 ± 30	146 ± 23	120 ± 40	115 ± 30
C_{max} (µg/ml)	0.06 ± 0.02	0.08 ± 0.01	0.07 ± 0.007	0.08 ± 0.02
$AUC_{0-\infty}$ (µg · min/ml)	17 ± 3	$39 \pm 7.0^{**}$	$26 \pm 2.0^*$	$32 \pm 9^*$
AUC_{0-last} (µg · min/ml)	16 ± 2	$28 \pm 5.0^*$	$22 \pm 1.6^*$	$28 \pm 9^*$
VZ _F (ml)	$50,642 \pm 2486$	$47,661 \pm 4490$	$47,708 \pm 5245$	$38,592 \pm 10690$
CL _F (ml/min)	296 ± 46	$131 \pm 21^{***}$	$193 \pm 15^*$	$161 \pm 50^{**}$
$AUMC_{0-\infty}$ (µg · min 2 /ml)	4024 ± 926	$17225 \pm 4230^{***}$	$7519 \pm 1289^*$	$10328 \pm 3424^*$
MRT $_{\infty}$ (min)	232 ± 20	$435 \pm 20^{***}$	$288 \pm 30^*$	$310 \pm 23^{**}$

VZ_F, volume of distribution; CL_F, clearance.

* $P < 0.05$, difference from control.

** $P < 0.01$.

*** $P < 0.001$ (difference from control).

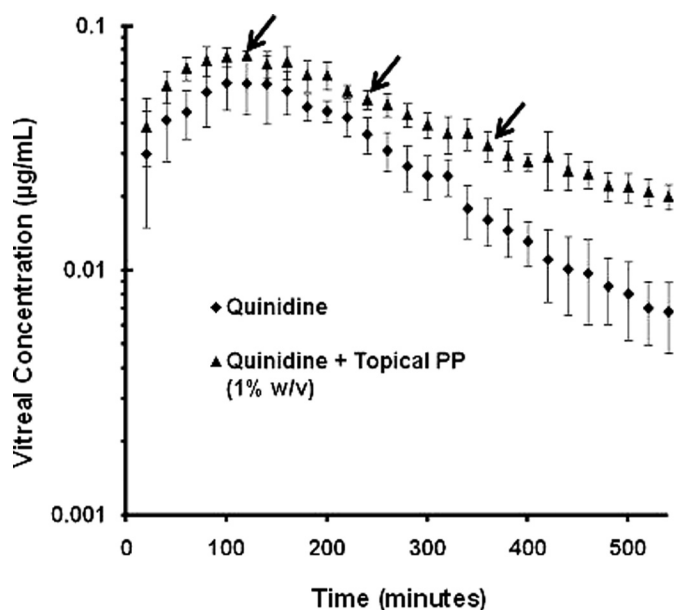


FIG. 2. Vitreal concentration-time profile of quinidine (5 mg/kg, systemic administration) alone (control) or in the presence of topically coadministered PP (1% w/v; 100 μ l administered at 2, 4, and 6 h after quinidine administration). Arrows indicate time of inhibitor administration. Data points represent mean \pm S.D. of four determinations. Control group data are the same as those in Fig. 1 and have been replotted to facilitate comparison.

information criterion and the Schwarz Bayesian criterion values (minimum for the two-compartment model compared with one- and three-compartment models). Therefore, a two-compartment open model was used to calculate the pharmacokinetic parameters. The plasma pharmacokinetic parameters of quinidine, such as elimination half-life, CL, AUC, k_{10} , k_{21} , k_{12} , steady-state volume of distribution, and apparent elimination rate constant remained unchanged in the presence of the topically coadministered inhibitors (Table 2).

Ocular Tissue Distribution of PP and Erythromycin after Topical Administration. Table 3 presents concentrations of PP and

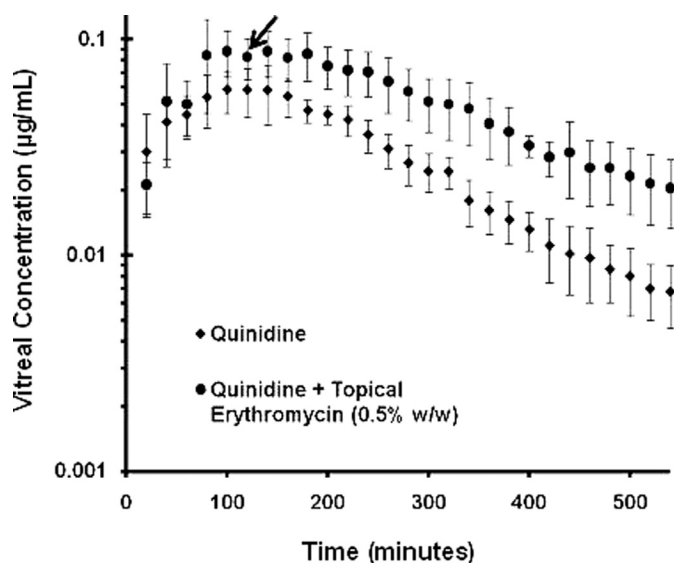


FIG. 3. Vitreal concentration-time profile of quinidine (5 mg/kg) alone (control) or in the presence of topically coadministered erythromycin ophthalmic ointment (0.5% w/w; 100 mg, administered 2 h after quinidine administration). Arrows indicate time of inhibitor administration. Data points represent mean \pm S.D. of four determinations. Control group data are the same as those in Fig. 1 and have been replotted to facilitate comparison.

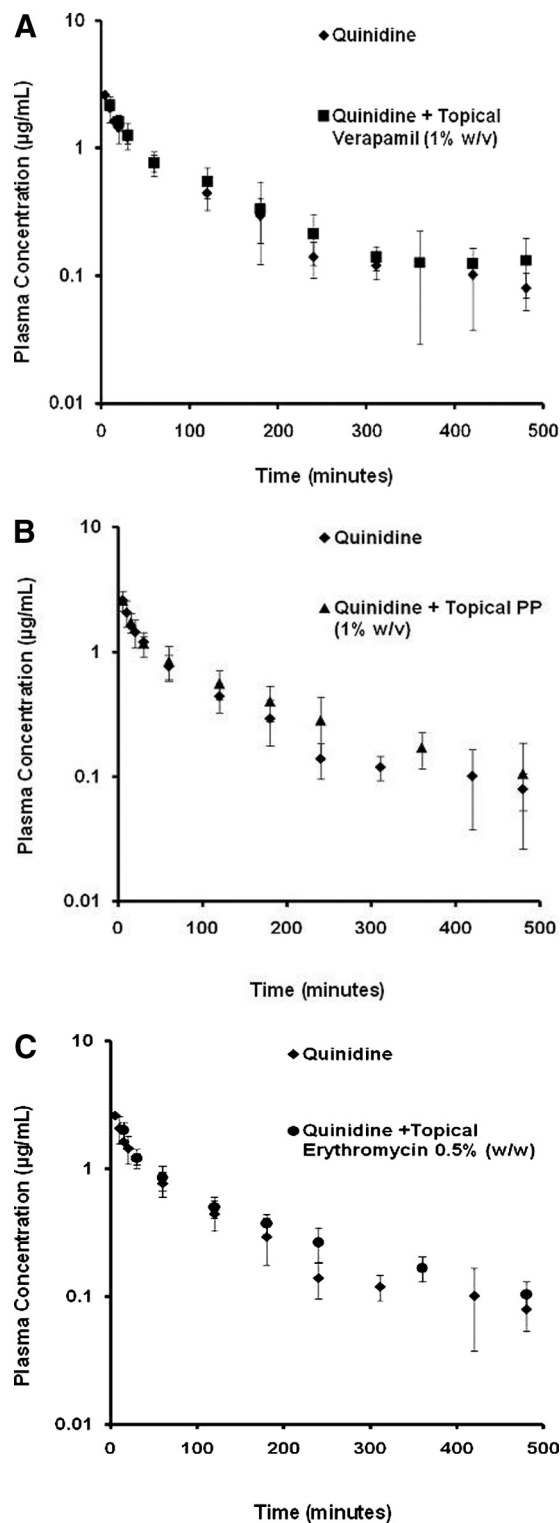


FIG. 4. A, plasma-time profile of quinidine (5 mg/kg, systemic administration) alone (control) or in the presence of topically coadministered verapamil 1% w/v (100 μ l administered at 2, 4, and 6 h after quinidine administration). Data points represent means \pm S.D. of four determinations. B, plasma-time profile of quinidine (5 mg/kg, systemic administration) alone (control) or in the presence of topically coadministered Prednisolone sodium phosphate (1% w/v; 100 μ l administered at 2, 4 and 6 h after quinidine administration). Data points represent means \pm S.D. of four determinations. C, plasma-time profile of quinidine (5 mg/kg, systemic administration) alone (control) or in the presence of topically coadministered erythromycin ophthalmic ointment (0.5% w/w; 100 mg administered 2 h after quinidine administration). Data points represent means \pm S.D. of four determinations.

TABLE 2

Plasma pharmacokinetic parameters of systemically administered quinidine (5 mg/kg) alone or in the presence of topically coadministered verapamil, prednisolone sodium phosphate ophthalmic solution (USP, 1% w/v), and erythromycin ointment

Verapamil and prednisolone sodium phosphate were administered at 2, 4, and 6 h after systemic quinidine administration. A single dose of erythromycin ophthalmic ointment (100 mg) was applied at 2 h after quinidine administration. No significant differences in the parameters were observed. A single control group (quinidine) was compared with each treatment group. Values represent means \pm S.D. ($n = 4$).

Kinetic Parameters	Quinidine	Quinidine + Verapamil (1% w/v)	Quinidine + Prednisolone (1% w/v)	Quinidine + Erythromycin (0.5% w/w)
k_{10} (min^{-1})	0.015 ± 0.003	0.015 ± 0.007	0.015 ± 0.002	0.027 ± 0.02
k_{12} (min^{-1})	0.021 ± 0.015	0.027 ± 0.011	0.040 ± 0.009	0.047 ± 0.04
k_{21} (min^{-1})	0.024 ± 0.016	0.020 ± 0.007	0.031 ± 0.015	0.02 ± 0.004
AUC ($\mu\text{g} \cdot \text{min}/\text{ml}$)	210 ± 20	233 ± 65	235 ± 54	231 ± 35
$k_{10} t_{1/2}$ (min)	52 ± 21	52 ± 20	48.5 ± 9	37 ± 15
CL (ml/min)	25 ± 3	23 ± 8	22 ± 5.5	22 ± 4
MRT $_{\infty}$ (min)	144 ± 58	170 ± 63	189 ± 44	142 ± 33
V_d (ml)	3756 ± 1636	3588 ± 487	3719 ± 1314	3196 ± 1276
β (min^{-1})	0.006 ± 0.001	0.0051 ± 0.002	0.0055 ± 0.001	0.0057 ± 0.001
$\beta t_{1/2}$ (min)	135 ± 83	142 ± 53	134 ± 38	124 ± 23

TABLE 3

Ocular distribution of verapamil, prednisolone sodium phosphate, and erythromycin ophthalmic ointment after topical administration

Prednisolone sodium phosphate (100 μl of a 1% w/v solution applied 2, 4, and 6 h after systemic administration) and erythromycin (100 mg of 0.5% w/w ointment applied at 2 h after systemic administration) tissue concentrations were determined 9 h after topical administration. PP values are reported for both intact PP and free prednisolone concentrations observed. Verapamil tissue concentrations were determined 7 h after topical administration (100 μl of a 1% w/v solution applied 2, 4, and 6 h after intravitreal quinidine administration). Values represent means \pm S.D. ($n = 4$).

Tissues	Topical Application of 1% w/v Verapamil ^a	Topical Application of 0.5% w/w Erythromycin	Topical Application of 1% w/v PP	
			Intact PP Conc.	Conc. of Free Prednisolone ^b
Aqueous humor ($\mu\text{g}/\text{ml}$)	7.9 ± 3.2	4.7 ± 1.0	11 ± 5.4	76.0 ± 2.0
Iris-ciliary body ($\mu\text{g}/\text{g}$)	4.4 ± 1.6	1.53 ± 0.31	4.1 ± 0.6	32.0 ± 7.0
Lens ($\mu\text{g}/\text{g}$)	6.3 ± 1.2	0.26 ± 0.04		16.0 ± 4.0
Vitreous humor ($\mu\text{g}/\text{ml}$)	0.086 ± 0.003	1.4 ± 0.15	1.8 ± 0.01	15.0 ± 2.0
Retina-choroid ($\mu\text{g}/\text{g}$)	47.0 ± 8.1	1.74 ± 0.05	3.0 ± 1.2	43.0 ± 10.0

^a Values from our previous study (Majumdar et al., 2009).

^b Generated as a result of bioreversion of PP in the ocular tissues.

TABLE 4

Apparent first-order degradation rate constants and half-lives of PP in ocular tissue homogenates (1 mg/ml protein content)

Values represent means \pm S.D. ($n = 4$).

Drug/Kinetic Parameters	Control	Aqueous Humor	Vitreous Humor	Iris-Ciliary Body	RPE/Choroid
k ($\times 10^3 \text{ min}^{-1}$)	0.36 ± 0.03	22.5 ± 4.0	0.83 ± 0.02	2.9 ± 0.09	3.17 ± 0.05
$t_{1/2}$ (min)	1933 ± 160	31 ± 6.0	835 ± 17.0	239 ± 8.0	218 ± 4.0

erythromycin in the aqueous humor, vitreous humor, and other ocular tissues at the end of the experiment (9 h). Topical administration of PP generated significantly high concentrations of prednisolone (generated from PP by enzymatic action) in the ocular tissues. Erythromycin concentrations in all the ocular tissues were significantly lower compared with those of PP. However, significant levels of erythromycin were observed in the vitreous humor even at 7 h after application. The lower concentrations of erythromycin generated in the ocular tissues compared with that of PP may be due to the differences in the dosing regimen. It should be noted that only a single dose of erythromycin, 100 mg of the 0.5% ointment, was applied 2 h after quinidine administration, whereas in the case of verapamil and PP, 100 μl of a 1% w/v solution was applied at 2, 4, and 6 h after quinidine administration.

Enzymatic Conversion of PP to Prednisolone. Tissue homogenates were prepared in IPBS (pH 7.4). Vitreous and aqueous humors were used as such. PP was rapidly hydrolyzed to the parent drug, prednisolone, by the ocular phosphatases. Table 4 depicts the apparent first-order degradation rate constants and half-lives of PP in the ocular tissue homogenates (1 mg/ml protein content), including aqueous and vitreous humor. Degradation rate constants were obtained from the log concentration of PP remaining versus time plots. The hydrolysis

rate constants obtained from the control (PP in IPBS) were subtracted from the overall observed rate constants to estimate rate constants for the enzyme-mediated hydrolytic process.

Discussion

The role of RPE P-gp in limiting the penetration of systemically or periocularly administered substrates into the posterior chamber of the eye is well established (Majumdar et al., 2009). We were surprised to find that very few studies have focused on strategies to overcome the efflux activity of this RPE P-gp. Duvvuri et al. (2003a) reported inhibition of the RPE P-gp with the help of an intravitreal inhibitor. In another in vivo study, Senthikumari et al. (2008a) demonstrated that systemically administered P-gp inhibitors significantly altered the ocular tissue concentrations of an intravitreally injected P-gp substrate. However, both routes of inhibitor administration have major drawbacks such as concerns associated with intravitreal injections and nonspecific systemic exposure of the inhibitor from intravenous administration and the limited, clinically relevant, inhibitor dose that can be administered (Majumdar et al., 2009).

In our previous study exploring the feasibility of a novel approach for inhibiting the RPE P-gp, we demonstrated that topical inhibitors could decrease the elimination rate of intravitreally administered P-gp

substrates (Majumdar et al., 2009). In the present study, we carried this investigation further to examine the effect of administration of a topical substrate/inhibitor on the ocular penetration of a systemically administered P-gp substrate. Although systemic administration of ophthalmic drugs also poses nonspecific systemic exposure concerns, this novel topical substrate/inhibitor coapplication approach could help decrease the systemic dose necessary to achieve an ocular therapeutic response.

The C_{\max} and T_{\max} of quinidine in the vitreous humor were observed to be $0.06 \pm 0.02 \mu\text{g/ml}$ and $115 \pm 30 \text{ min}$, respectively. The vitreal AUC of quinidine was found to be 8% of the systemic AUC (Tables 1 and 2). These results are consistent with previous literature reports (Duvvuri et al., 2003a). Coadministration of topical verapamil and PP at 2, 4, and 6 h after systemic (intravenous) quinidine administration resulted in a significant decrease in the apparent vitreal λ_z and in an increase in the vitreal $t_{1/2}$ and MRT of quinidine (Table 1; Figs. 1 and 2). Consistent with this result, erythromycin also produced a significant decrease in the vitreal λ_z and an increase in the vitreal $t_{1/2}$ and MRT of quinidine (Table 1; Fig. 3). These findings strongly suggest that topically coadministered P-gp inhibitors interact with the RPE P-gp, resulting in decreased vitreal elimination of the systemically administered P-gp substrate.

Duvvuri et al. (2003a) reported a significant increase in the C_{\max} and the rate of entry of quinidine into the vitreous and a significant decrease in the T_{\max} of quinidine after systemic administration of quinidine and intravitreal administration of the inhibitor. However, in the present study a statistically significant difference in the C_{\max} and T_{\max} of quinidine in the vitreous humor, on topical coadministration of the three inhibitors, was not observed (Table 1). This result may be explained by the fact that Duvvuri et al. (2003a) administered the intravitreal inhibitor 20 min before systemic quinidine administration, whereas in the present study the inhibitors were applied 2 h after quinidine administration, by which time the vitreal quinidine absorption phase would have been over.

Significant differences in the central compartment pharmacokinetic parameters of quinidine in the presence and absence of topically coadministered verapamil, PP, or erythromycin were not observed (Table 2; Fig. 4). These findings strongly suggest that the quinidine doses administered across groups were uniform and that the observed effect of the topically coadministered verapamil, PP, and erythromycin on the vitreal kinetics of quinidine was a result of modulation of the efflux activity of RPE P-gp.

Topically coadministered verapamil produced a 1.7-fold decrease in the terminal elimination rate constant of intravitreally administered quinidine, whereas $\text{AUC}_{0-\infty}$ and CL remained unchanged (Majumdar et al., 2009). Here, however, verapamil, with a similar dosing regimen, generated a greater (2.2-fold) decrease in the elimination rate constant of quinidine from the vitreous humor and also a significant increase in the vitreal $\text{AUC}_{0-\infty}$ and a decrease in CL. These are probably due to lower quinidine concentrations generated at the P-gp binding site in the RPE cytosol on intravenous administration. At the end of 2 h, when the topical inhibitors were administered, the free plasma quinidine concentration was $0.05 \mu\text{g/ml}$ [considering almost 80 to 90% protein binding (Guentert and Oie, 1980; Ochs et al., 1980)], and the free vitreal quinidine concentration achieved was $0.06 \mu\text{g/ml}$ after systemic administration (current study), whereas the vitreal free concentration of quinidine was $0.3 \mu\text{g/ml}$ (as estimated using microdialysis) after intravitreal injection. If we assume that similar verapamil concentrations were generated at the RPE/choroid after topical administration in both studies, the verapamil/quinidine ratio would be significantly higher at the RPE P-gp binding site when quinidine is administered systemically. This results in greater inhibition and thus

a significant decrease in the CL and elimination rate constant and a significant increase in the vitreal $\text{AUC}_{0-\infty}$ in the presence of verapamil compared with the effects of intravitreal administration (Majumdar et al., 2009).

Interestingly, 1% w/v PP produced a greater decrease in the vitreal λ_z compared with 1% w/v prednisolone hemisuccinate sodium (PHS) in our earlier study (Majumdar et al., 2009). In addition, a statistically significant difference in the $\text{AUC}_{0-\infty}$ and CL of quinidine was also observed in the presence 1% w/v PP. This can be explained by the low concentrations of quinidine generated in the vitreous humor (after systemic administration), by the rapid bioreversion rates of PP to prednisolone, and by the formulation components of the PP ophthalmic solution. Prednisolone and not PP is known to interact with P-gp (Majumdar et al., 2009). In vitro metabolism studies confirmed faster bioreversion of PP to prednisolone in the RPE/choroid ($t_{1/2}$ 218 min) compared with that of the hemisuccinate ester (PHS) ($t_{1/2}$ 578 min) (Majumdar et al., 2009), possibly as a result of differences in the phosphatase and esterase levels in the ocular tissues (Attar et al., 2005).

It is also interesting to note that topical PP (1%) generated significantly higher concentrations of prednisolone ($15.0 \pm 2.0 \mu\text{g/ml}$) in the vitreous humor compared with the vitreal prednisolone levels achieved with topical 2% PHS ($0.49 \pm 0.29 \mu\text{g/ml}$) (Majumdar et al., 2009). Moreover, 2% PHS generated only $16.7 \pm 1.6 \mu\text{g/g}$ prednisolone in the RPE/choroid tissue (Majumdar et al., 2009), whereas 1% w/v PP generated $43 \pm 10 \mu\text{g/g}$ (Table 3) of prednisolone. The higher levels of prednisolone obtained in the posterior segment ocular tissues support our earlier observation that hydrophilic moieties on topical application tend to permeate to the back-of-the-eye tissues better (Majumdar et al., 2009). In addition, the PP formulation used in this study contains benzalkonium chloride (BAK) (0.01% w/v) and EDTA as formulation components. The role of BAK and EDTA in enhancing corneal, conjunctival, and scleral permeation of compounds is well documented (Scholz et al., 2002; Okabe et al., 2005; Majumdar et al., 2008). Thus, PP might have diffused across the conjunctiva and into the sclera much more efficiently, generating higher PP and prednisolone concentrations in the RPE/choroid. The significantly higher PP and prednisolone concentrations in the aqueous humor, lens, and iris-ciliary body also support enhanced transcorneal PP permeation in the presence of BAK and EDTA and rapid generation of prednisolone in the aqueous humor. Prednisolone, being very lipophilic, would then rapidly partition into the anterior segment ocular tissues and then laterally migrate along the iris-ciliary body to the sclera and then to the RPE and vitreous humor. Thus, overall, the results suggest that BAK and EDTA enhanced penetration of PP into the back-of-the-eye tissues.

The levels of verapamil (approximately $0.2 \mu\text{M}$) obtained in the vitreous humor (1 h after the last dose) (Majumdar et al., 2009) were much lower compared with those of PP (approximately $3.7 \mu\text{M}$) and prednisolone (approximately $41 \mu\text{M}$) in the vitreous humor even at 3 h after the last dose. Although similar concentrations of prednisolone ($43 \pm 10.0 \mu\text{g/g}$; approximately $120 \mu\text{M}$) and verapamil ($47 \pm 8.1 \mu\text{g/g}$; approximately $95 \mu\text{M}$) (Majumdar et al., 2009) were generated at the RPE/choroid in both studies, the extent of RPE P-gp inhibition was less with prednisolone than with verapamil (Table 1), probably because verapamil is a more potent P-gp inhibitor (prednisolone IC_{50} is $300 \mu\text{M}$ compared with IC_{50} of $4.7 \mu\text{M}$ for verapamil; a 65-fold difference) (Schwab et al., 2003; Hariharan et al., 2009).

Furthermore, in our previous study, erythromycin, at a concentration of 0.2% w/v, applied at 2, 4, and 6 h after intravitreal quinidine administration did not significantly affect the pharmacokinetic param-

eters of quinidine (Majumdar et al., 2009). On the contrary, in the present investigation erythromycin ophthalmic ointment produced a statistically significant difference in the vitreal λ_z , $AUC_{0-\infty}$, CL, and MRT of systemically administered quinidine. A single application of the ointment, 100 mg of 0.5% w/v applied 2 h after systemic quinidine administration, produced significant levels of erythromycin in the vitreous humor and RPE/choroid, even 7 h after erythromycin application, suggesting depot formation and sustained release of the drug from the residual formulation in the cul-de-sac. The observed effect on quinidine pharmacokinetics can probably be explained by the higher ocular tissue concentrations of erythromycin achieved in this study and the low concentrations of free quinidine in the plasma and RPE (as discussed earlier) and thus a higher erythromycin/quinidine ratio in the RPE P-gp microenvironment.

Besides P-gp, the RPE is known to express other transporters (e.g., multidrug-resistant proteins and organic anion-transporting polypeptides) (Hughes et al., 2005) with which quinidine may interact (Makhey et al., 1998; Vezmar and Georges, 2000; Shitara et al., 2002). Although quinidine is a preferred substrate of P-gp and has been widely used as a P-gp substrate/inhibitor (Kusuhara et al., 1997; Duvvuri et al., 2003a; Jain et al., 2004; Suzuki et al., 2007), some involvement of other transporters cannot be ruled out completely. Future studies to optimize this approach will involve more specific P-gp/multidrug-resistant protein inhibitors and substrates. In addition, the effect of inhibitor type, concentration, time of application, and time course of inhibitors in the vitreous, plasma, and other ocular tissues after topical application on ocular kinetics of systemically/intravitreally administered P-gp substrates will also be the object of consideration in our future studies.

In conclusion, this study demonstrates for the first time that topically applied P-gp inhibitors can diffuse to the RPE and alter the elimination kinetics of a systemically administered P-gp substrate, probably through inhibition of the RPE P-gp. The degree of inhibition will depend on the physicochemical characteristics of the inhibitor and its affinity for P-gp and the concentration of the therapeutic agent in the plasma or vitreous humor. Formulation factors such as inclusion of permeation and viscosity enhancers may play a major role in yielding effective levels of the inhibitor at the RPE. This interaction may be used positively for drug delivery purposes through the use of potent, pharmacologically inactive, efflux inhibitors.

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